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Isolation of Symbiotically Expressed Genes From the Dinoflagellate Symbiont of the Solitary Radiolarian *Thalassicolla nucleata*

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Symbiotic associations are fundamental to the survival of many organisms on Earth. The ability of the symbiont to perform key biochemical functions often allows the host to occupy environments that it would otherwise find inhospitable. This can have profound impacts upon the diversification and distribution of the host. Cellular organelles (chloroplasts and mitochondria) represent the final stages of integration of endosymbionts. These organelles were of critical importance to the evolution and success of eukaryotic lineages on our planet because they allowed the host cells to harness light energy and to thrive in the presence of oxygen. The marine photosymbiotic associations that we study represent an earlier stage in the process of symbiont integration—one in which the photobiont can still be removed from the host and exist on its own. These systems are of interest to us for two reasons. First, they are ecologically important in the marine environment where they occur. These organisms form zones of photosynthetic production in oceanic regions typically low in nutrients. Second, investigation of these interactions may shed light on the molecular and evolutionary mechanisms involved in the integration of cells and their genomes.

Associations between microbial symbionts and their hosts are fundamental trophic relationships that occur in both terrestrial and aquatic environments and depend upon regulated genomic communication between the interacting organisms. We are particularly interested in the photosymbiotic relationships that involve microscopic (protistan) algae in association with a variety of "invertebrate" hosts (protistan and metazoan); these relationships are fairly common in the marine environment and contribute significantly to nutrient flow in the nutrient-poor regions of the ocean where they occur. The symbiosis is essential to the survival of the host organisms, although the algal symbiont can exist in the free-living state.

Besides the conspicuous invertebrate photosymbioses that most people are familiar with (anemones and corals), a variety of protistan species also harbor cytoplasmic algal symbionts. Most notably, protozoa within the subphylum Sarcomastigophora (the foraminifera, radiolaria, and acantharia; collectively referred to as "sarcodine protozoa") exist in symbioses with a wide array of algae (Anderson, 1983; Lee and McEnery, 1983; Caron and Swanberg, 1990; Michaels, 1991). These sarcodine protozoa are often large for single-celled organisms. Solitary cells often attain sizes more than 1 mm in diameter (some benthic foraminifera form discs more than 1 cm in diameter), and the presence of complex pseudopodial networks can increase the effective size of these organisms up to a few centimeters (Fig. 1). Some colonial species of radiolaria even form gelatinous pseudopodial matrices several centimeters in diameter and more than 1 m in length. These organisms are important contributors to the biological assemblages in all tropical and subtropical oceans of the world. Sarcodines form conspicuous zooplankton assemblages in oligotrophic surface waters, where they contribute significantly to primary productivity (by virtue of their intracellular symbionts), predation,

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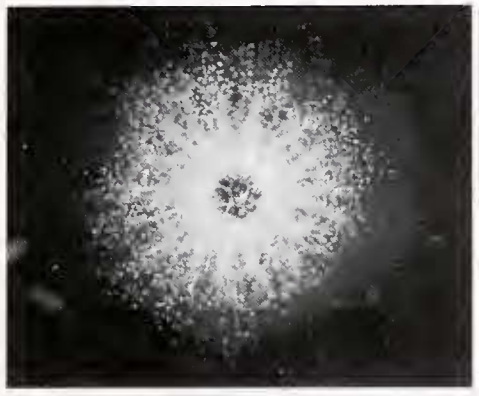

Figure 1. Solitary planktonic radiolarian *Thalassicolla nucleata*. The central capsule of the organism is the dark circle in the center; it is surrounded by the rhizopodia containing the symbionts (small dots around the central capsule). The entire organism is about a millimeter in diameter.

and the vertical flux of organic material and crystal minerals, such as calcium carbonate and strontium sulfate (Caron and Swanberg, 1990; Swanberg and Caron, 1991; Caron *et al.*, 1995; Michaels *et al.*, 1995).

Our interest in the regulation of photosymbiotic relationships arose while working on the identification of the symbionts in planktonic foraminifera and radiolaria to determine whether co-evolution of host and symbiotic alga occurred (Gast and Caron, 1996; Gast *et al.*, 2000). We molecularly identified several taxonomically different algae that form symbioses in the planktonic sarcodines. Dinoflagellates are the most common of these, but we also found prymnesiophyte and prasinophyte algae. Furthermore, it appears that most algal symbionts are actually genetically more similar to *non*-symbiotic algae than they are to other symbiotic algal lineages. This led us to wonder about what made very different algae suitable as symbionts, sometimes in the same host (Gast and Caron, 2001), and to hypothesize that symbiotic algae have something in common, which can be identified genetically, that makes them acceptable as symbionts. We have undertaken comparative studies of gene expression in a symbiotic alga to investigate the mechanisms of symbiotic communication.

We are studying the symbiont-host relationship between the dinoflagellate alga *Scrippsiella nutricula* and the solitary planktonic radiolarian *Thalassicolla nucleata*. The radiolarian system allows us to compare the genes expressed in the symbiotic state of the algal symbiont with those expressed in the free-living state of the alga in culture. The information that we obtain from this system can be used to identify the genes necessary for maintaining the interaction, determine the timing of gene expression, examine the genomic characteristics associated with symbiotic-competence (why one alga is a good symbiont and another is not), and examine the mechanisms of secondary and higher level endosymbiotic events.

We chose to begin our work of isolating algal genes expressed in the symbiotic state by studying the dinoflagellate symbiont (*Scrippsiella nutricula*) of the solitary radiolarian *T. nucleata* (Fig. 1). To identify genes being expressed in the symbiosis, we required RNA from both the free-living and symbiotic states of the alga. We currently have the different algal symbionts as free-living laboratory cultures, but the intact association must be collected from the environment. *T. nucleata* generally harbors thousands of symbiotic algae, it can be collected from the environment fairly easily, and, most importantly, the host and symbiont can be separated from each other with minimal host RNA contamination of the sample. The host nucleus, organelles, and ribosomes are separated from the symbionts by the central capsule (pp. 90–94, Anderson, 1983). We believe that very little host mRNA will be present in the extracapsular material, and we are careful not to destroy the central capsule when stripping away the desired extracapsular matrix.

Thalassicolla nucleata was collected by plankton net tows in the Sargasso Sea and the San Pedro Channel. Individuals were transferred to sterile seawater in multiwell culture dishes and placed in lighted incubators or on workbenches at ambient temperatures. This allowed the hosts to clear themselves of prey items prior to recovery of the symbiont. All hosts were examined for prey before the extracapsular material was stripped away from the central capsule. Studies involving host incorporation of ^{14}C through the uptake of symbiont-produced photosynthate indicated that the interaction continued to function normally when the host was held in the laboratory (Anderson, 1978; Gast, unpubl. results). We believe that the symbiotic pattern of gene expression is also maintained. Stripped extracapsular material was immediately placed in RNALater (Ambion) until processed for RNA extraction (RNAqueous, Ambion). At least 200 individual hosts were collected and processed for each subtraction analysis.

Due to the relatively small amount of symbiotic-state RNA available for our project, we used a subtraction method that would permit the analysis of very small amounts of starting material. Through the use of two PCR enrichment amplifications, suppression subtractive hybridization (SSH; Diatchenko *et al.*, 1996) allows as little as 25 ng of mRNA (or 50 ng of total RNA) to be analyzed. We used the Clontech PCR-Select cDNA Subtraction Kit to analyze both total RNA and mRNA from the dinoflagellate symbionts (Fig. 2). To date, we have completed five subtractions—one from total RNA that failed, three from total RNA that were successful but yielded mostly host and bacterial ribosomal genes, and one from mRNA that was successful. We switched to analyzing mRNA because we recovered as many ribosomal clones as symbiosis clones when we analyzed total RNA. Contaminating ribosomal fragments were not unexpected since they would not be

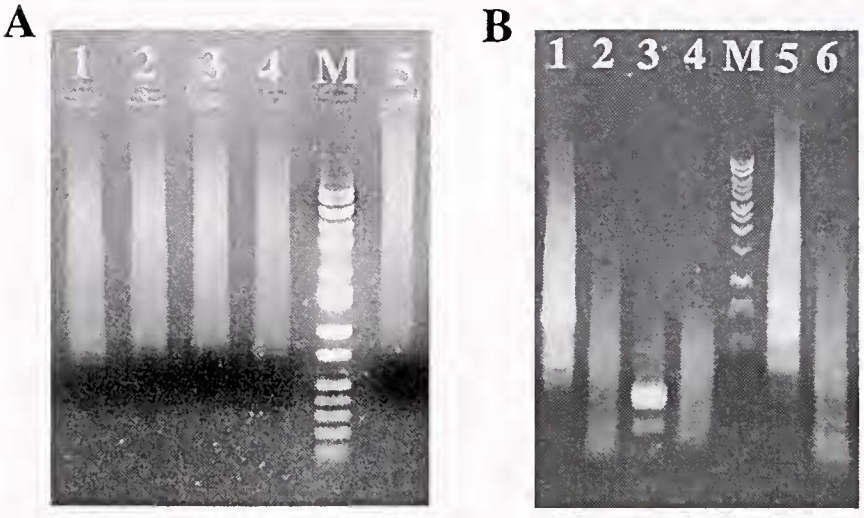

Figure 2. Results from suppression subtractive hybridization. (A) cDNA synthesis from symbiotic (lanes 1 & 2), free-living (lanes 3 & 4), and control (lane 5) RNA. M = 1-kb ladder. (B) Final PCR products from subtracted samples. Lane 1—symbiotic, subtracted; lane 2—symbiotic unsubtracted; lane 3—free-living subtracted; lane 4—free-living unsubtracted; lane 5—control subtracted; lane 6—control unsubtracted. M = 1-kb ladder. cDNAs were generated and subtracted by using the PCR-Select cDNA Subtraction kit from Clontech. Total RNA was isolated from the algae in the symbiotic state and the free-living state using either the Ambion RNAEasy kit or the Epicentre MasterPure RNA Purification kit, and mRNA was recovered using the Qiagen Oligotex mRNA kit. In both instances, the RNA from each state was separately reverse transcribed using MMLV-reverse transcriptase (Superscript II) to generate double-stranded cDNAs. Both sets of cDNAs were restriction digested with *Rsa*I to generate fragments with blunt ends, and the digested cDNAs from the symbiotic state were divided into two aliquots. Each aliquot was ligated to one of two different adaptors supplied in the kit. The strategy of the forward subtraction is to identify cDNAs up-regulated or unique to the symbiotic state of the alga. In this process, an excess of cDNAs from the free-living state (without ligated adapters) was mixed in two separate reactions with the adaptor-ligated cDNAs from the symbiotic state. The two tubes were separately heated to 95°C to denature the cDNAs, and then each was allowed to hybridize at 68°C for 8 h. This step promotes hybridization between the cDNAs expressed in common in the symbiotic and free-living states. To further enhance the subtraction of common cDNAs, the contents of the two tubes were then mixed together without a second denaturation step, combined with an additional excess of heat-denatured cDNAs from the free-living state, and hybridized overnight at 68°C. PCR primers specific to the two adapters (Clontech) were used to amplify cDNAs created during the subtraction that possessed one DNA strand ligated to adapter 1 and the other DNA strand ligated to adapter 2R. Only cDNAs from the symbiotic state of the alga should be able to amplify in this step, so we are enriching for the differentially regulated transcripts. The subtracted and amplified cDNAs were cloned into pGEM-T (Promega) and used to transform JM109 competent cells (Promega). A reverse subtraction was also performed in which the adaptor-free cDNA was from the symbiotic state and the adaptor-ligated cDNAs had been isolated from the free-living state. These subtracted cDNAs are therefore enriched in genes specific to the free-living state.

removed by subtraction with cultured dinoflagellate RNA. We identified 23 clones as being potentially up-regulated in the symbiotic state of the alga.

Nucleotide Blast searches (Altschul *et al.*, 1997) were conducted for sequences from each of the putatively up-regulated clones that were isolated. Of the 23 selected clones, 8 turned out to be ribosomal genes from the host, bacteria, or fungi. Twelve of the remaining clones (C4 group) were very similar to each other, varying primarily by small differences in length. These clones had no significant sequence match in the database (Table 1). Of the remaining three clones, only F7 showed significant homology to database sequences. The homology between F7 and Cyplasin S is the result of three 30-base repeats in the clone sequence that are also present in the gene. This 30-base repeat seems to occur in other proteins as well, and may represent a shared structural region. There are generally very few algal or protist cDNA sequences in GenBank, so the overall lack of homology to sequences in the database is disappointing but not unexpected. We are continuing to screen clones from the final PCR products of our mRNA subtraction to obtain more genes unique to the symbiotic state of the alga.

Because of the small amount of RNA available from our symbiotic state, it was not possible to confirm differential gene expression using northern blot. Instead, we used cDNA generated from the free-living and symbiotic state RNAs in a dot blot format (Fig. 3). Clone F9 was included as a control for positive hybridization to the free-living

Table 1

List of clones recovered and analyzed with suppression subtractive hybridization

Clone group	BLASTn results	E value	Number of clones	Size of fragment (bp)
C4	Human DNA clone RP11-100A16	0.049	12	278
F7	*Aplysia punctata* mRNA for Cyplasin S	1e-60	1	161
E7	*Klebsiella pneumoniae* SL032 plasmid	0.003	1	635
B8	Human chromosome 16 clone RP11-407623	0.095	1	367

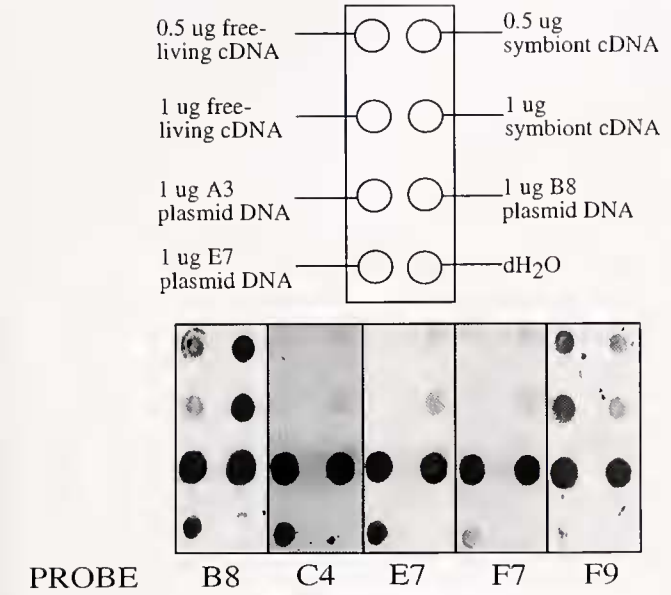

Figure 3. Confirmation of clone expression in symbiotic cDNA. To determine if the subtracted library contained clones up-regulated in the symbiotic state, 96 colonies were randomly chosen, and the cDNA insert from each was PCR amplified with the adapter-specific primers. Amplified products were denatured by addition of an equal volume of 0.6 *N* NaOH and then spotted onto Hybond NX nylon membranes and baked at 80°C for 1 h. The membrane was then hybridized to either a forward-subtracted or reverse-subtracted cDNA probe. Probes were generated by incorporating biotin-labeled nucleotides into either the secondary PCR products or the original cDNAs. Membranes were prehybridized for 1 h at 65 or 72°C in hybridization buffer (5XSSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking powder). The denatured probe was added to the prehybridization and allowed to hybridize overnight at 65 or 72°C. Membranes were washed twice, for 5 min each, in 2XSSC, 1.0% SDS at room temperature. The next two washes, 15 min each, were in 2XSSC, 0.1% SDS at the hybridization temperature. Hybridization was detected using the CDP-Star Southern Star chemiluminescent method. Only those inserts that showed hybridization to the forward-subtracted probe but not to the reverse-subtracted probe were examined further. To confirm that these clones were differentially regulated, inserts from these positive clones were PCR amplified and digested with *Rsa*I, *Sma*I, and *Eag*I to remove adaptors. Samples (0.5 and 1 mg) of the unsubtracted cDNAs from the symbiotic and free-living states, along with the plasmid clones A3, E7 and B8, were denatured, spotted onto membranes, and probed with the labeled inserts. The results for the symbiotically up-regulated clones (B8, C4, E7, and F7) are shown, along with one clone that is down-regulated in the symbiotic state (F9).

cDNA. This and other clones represent potentially down-regulated genes in the symbiotic state of the alga. We have not begun to analyze these clone sequences, but we recognize that these are potentially informative as well. The dot blots allowed us to confirm that our differentially expressed clones were not expressed, or were expressed at a reduced level, in the free-living cDNA populations.

Suppression subtractive hybridization analysis generates fairly small cDNA fragments, so rapid amplification of cDNA ends (RACE) analyses must be used to obtain the full cDNA sequence. We began our RACE analysis with the B8 clone because we knew that we had the 3′ end of the transcript. Currently we have about 800 basepairs of sequence for this clone. It has two open reading frames that are interrupted by a single stop codon in each. Neither of these reading frames has a start codon, and because the few dinoflagellate cDNAs that have been examined indicate the presence of fairly long 3′ untranslated regions (300–500 basepairs), we may still not have reached the 5′ end of the transcript. New primers are being designed to try and recover more of the 5′ end. Our other clones are less defined as to whether they are at the 5′ or 3′ end of the cDNA, and we are currently extending from both ends.

In this decidedly ambitious project, we have been successful in recovering several genes that appear to be up-regulated in the symbiotic state of the dinoflagellate symbiont *S. nutricula*. We know of only one other study using SSH in a non-mammalian context. That study, on the sexual stages of diatoms (Armbrust, 1999), confirmed only 10 up-regulated clones, so our low number of recovered clones may not be unusual, especially since our RNA quantities were so limiting. We plan to acquire the full-length cDNA sequences for the clones we have; to pursue the construction of a cDNA library for the dinoflagellate symbiotic-state RNA; and to develop microarrays to aid in screening for differentially expressed genes.

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